MECHANISM OF ENZYMATIC ADAPTATION IN GENETICALLY CONTROLLED YEAST POPULATIONS

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Introduction.—For some time now it has been recognized that the fermentation of galactose by yeast (see Lippmann[1]) differs greatly from that of other hexoses. Armstrong[2] found that some yeasts had and others had not the power of fermenting galactose. Slator,[3] in a quantitative investigation of the same subject, was able to confirm the statement previously made, that certain yeasts which have the power of fermenting galactose, possess it only after the yeast had been acclimatized by culture in the presence of the sugar.

The nature of this acclimatization has been the subject of some investigation. The experiments of Söhngen and Coolhaas[4] would seem to indicate that the production of galactozymase parallels the formation of new cells. The results of Euler and Nilson[5] also lead to this conclusion. More recently, however, Stephenson and Yudkin[6] concluded from their experiments that the production of galactozymase in a yeast culture need not involve the formation of new cells. They interpret the phenomenon as a direct cytoplasmic reaction to the presence of the substrate (galactose) leading to the formation of a new enzyme. These authors used CO_2 evolution in a period when the population remained constant according to the total and viable counts.

It is clearly of some importance for the further elucidation of this acclimative or adaptive phenomenon to know whether or not new cell formation is involved in the appearance of a new physiological property in a culture. The data presented in this paper indicate that the conflict between the results and conclusions of Stephenson and Yudkin and those of previous workers cited, is more apparent than real. Both types of phenomena ("acclimatization" with and without the formation of new cells) can be observed depending upon the genetic background of the yeast strain being used.

Difficulties in Interpretation.—It must be recognized that microörganisms are handled in such large numbers that when a comparative biochemical investigation of microörganismic physiology is being made, over-all populational characteristics, in contradistinction to the properties of individuals, are being studied. The mechanisms available to an individual for adapting itself to an environmental change are limited by its genome and the physiological flexibility permitted by its particular degree of specialization. When, however, the adaptive ability of a population is being considered, there must be added to the physiological pliability of its members the genetic plasticity of the group in terms of the numbers and kinds of mutants it is capable of producing.

This composite nature of populational adaptability makes the interpretation of observed physiological changes in bacterial or yeast cultures difficult. In any particular case the same end-result can clearly be obtained by any one of the following: (1) the natural selection of existent mutants with the desired physiological characteristic from a heterogeneous population; (2) the presence of the substrate induces a "new"[7] enzymatic system in all the members of a homogeneous population, resulting in a gradual increase in the measured enzyme activity of the population; (3) a combination of natural selection and the action of mechanism (2) on those selected.

In most cases where so-called "training" has been observed to occur in microbic cultures, wherever a decision is at all possible, the most likely explanation seems to be that a natural selection of a variant type has taken place. Relatively few investigations (see Dubos,[8] Yudkin[9] and Karström[10]

for recent reviews) can lend much support to the hypothesis of a direct substrate-cytoplasmic interaction.

Thus far, those who have drawn the conclusion that mechanism (2) is operating have depended for the most part on demonstrating the existence of a constant total and viable count during the period when the physiological change in the population was being noted. A constant viable count alone would clearly have little critical value in deciding the question at issue. During the stationary phase of population growth new individuals must make their appearance to take the place of those that have died and natural selection could work in this period as well as in any other of the growth cycle.

However, the use of the constant *total* count involves some difficulties which may briefly be summarized as follows:

1. The failure to find "adaptation" in the complete absence of cell division would not necessarily be conclusive evidence that none exists. It is conceivable that in cultures where this "ideal" had been reached the physiological state of the cells was such that the ability to synthesize new enzymatic systems had been lost along with the ability to divide.

2. This method has the further disadvantage that it cannot distinguish between the following two situations:

(*a*) All the members of a phenotypically homogeneous population react to some component in the environment in an "adaptive" manner.

(*b*) Starting with a phenotypically heterogeneous population only those react which, due to a proper genetic constitution, already possess an adequate though latent enzymatic system. Under this condition providing the genetic variants formed a reasonably high proportion of the population (10% and up) an increased enzymatic activity with time would be observed without any concomitant cell division.

It is evident from the above discussion that the crucial point at issue in questions of this nature is the phenotypic homogeneity or heterogeneity of the starting population. And, unless in a particular case a decision is arrived at on this question, no precise or certain conclusions can be drawn, even under conditions of a constant total count, concerning the mechanism involved in accomplishing an observed change in the physiological characteristics of a population.

Since limitations of size, and sensitivity of available methods, prevent a direct examination of the individuals in a microörganismic population as a means of deciding the question of phenotypic homogeneity, recourse has been made to another method.

A cell can in general be characterized by the type of colony it produces when grown on an agar surface under standard conditions. Thus if a representative sample of a population is plated out and more than one colonial type is noted, it may be logically concluded that more than one type of in-

dividual is represented in the population. This method of examination can, under controlled conditions, yield quantitative data of a high degree of reproducibility and has been used to examine the kinetics of the appearance of morphological variants in bacterial cultures (see Shapiro, Spiegelman and Koster[11]).

Methods and Material.—(*A*) *Yeast Strains:* Two strains of *Saccharomyces cerevisiae* isolated in this laboratory and known as Db23B and LK2-G12 were used in the following experiments. Both strains could acquire the ability to ferment galactose if grown in its presence. Strain Db23B originated from a single ascospore and its population contains principally haploid cells and is therefore characteristically unstable. Strain LK2G12 originated from an intact 4-spored ascus in which copulation was observed to occur pairwise. Consequently it is known to be a diploid and in contrast to the Db23B is characteristically stable. For further details concerning yeast breeding and strain isolations and the implications of haploidy and diploidy for genetic stability consult Lindegren and Lindegren.[12]

(*B*) *Media:* The basic medium contains 2 g. of KH_2PO_4, 5 g. of peptone and 2.5 g. of dried dead yeast per liter. To this was added the desired carbohydrate. Agar plates were made by adding the requisite amount of agar to the above basic medium. It was found necessary to filter the agar medium to facilitate observation of the colonies.

(*C*) *Carbohydrates:* As sources of carbohydrate, reagent grade chemicals were used. Difco's purified galactose was further treated according to a method described by Stephenson and Yudkin[6] to remove any contaminating fermentable sugars.

(*D*) *Cell Count:* Cell counts were made by means of an haemocytometer.

(*E*) *Test Plates:* Since we are here concerned with whether or not the organisms can produce CO_2 from galactose, some method had to be devised which would permit the detection of this characteristic. The procedure adopted was to plate a sample of a population on an agar surface, containing 4% galactose purified as described above, and then cover it with another layer of galactose-containing agar. The production of gas cracks the agar right above the colony and thus gas producers can be distinguished easily from those which cannot evolve gas from this sugar. Figure 1 represents a typical field containing both positives and negatives.

To examine the type composition of a population growing on an ordinary glucose plate, well-isolated colonies incubated for 72 hrs. at 26°C. were used. These were removed from the agar surface and suspended in chilled galactose broth and then diluted to contain about 5000 cells per cc. as determined by direct count with an haemocytometer. Of this suspension, 0.2 cc. was then placed on a 4% galactose-4% agar surface from which all excess fluid had been allowed to drain. A sterile bent rod was then rotated over the surface to get even distribution. A 5% agar medium con-

taining 4% galactose cooled to 39° was poured over the inoculated surface. These test plates were incubated at 28°C. for at least 48 hrs. before count-

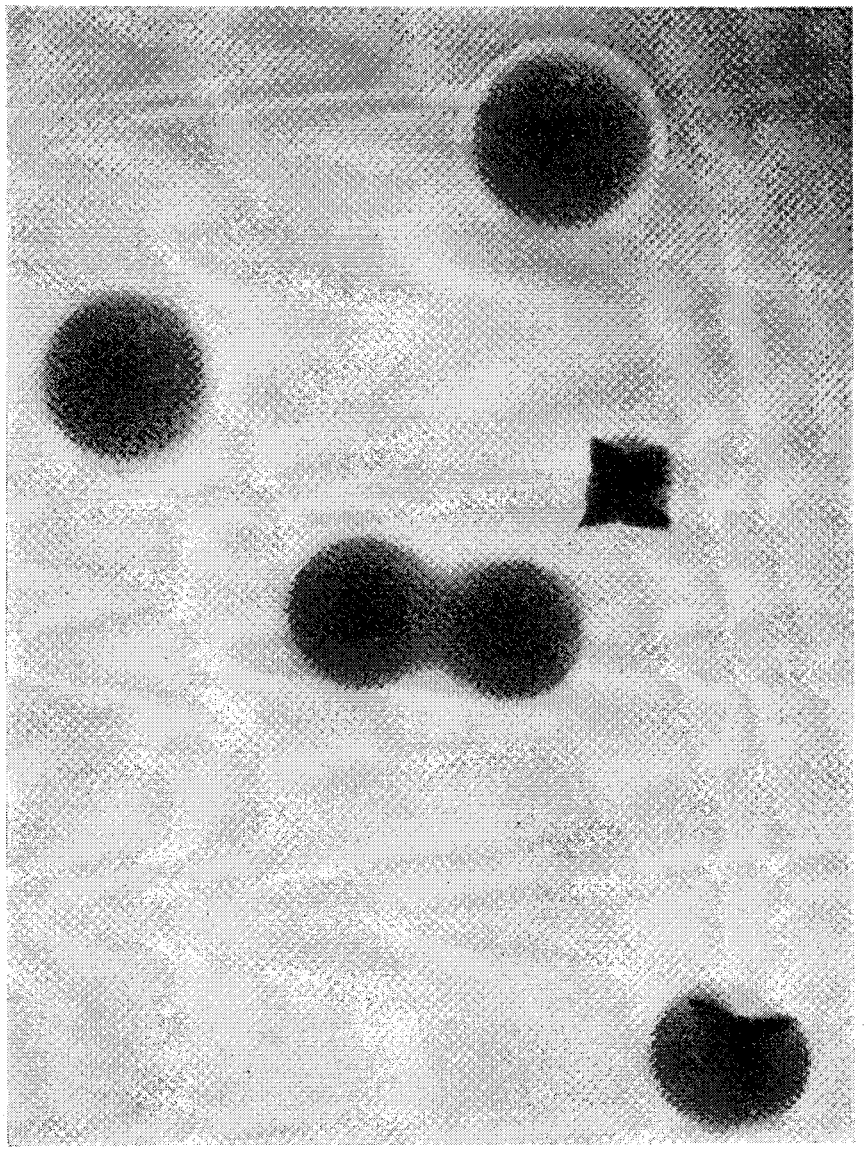

FIGURE 1

Field of a test plate in which colonies grow embedded between two layers of agar. Positives (gas producers) are easily distinguished by the typical star-shaped crack in the agar of its immediate neighborhood.

ing. Five such test plates were prepared from each suspension. Counting was done under the low power of a binocular dissecting microscope.

Experimental Results.—(*a*) *Data obtained from strain Db23B:* Table 1 records results of the galactose test-plate examination of the composition of 10 colonies of Db23B grown on glucose agar. It is clear that two types of individuals, at least as far as gas production from galactose is concerned, are initially present. The percentage of the population, grown on glucose, which is able to ferment galactose, varies from 2.0 to 15.0%.

TABLE 1

COMBINED TABLE SHOWING RESULTS OF EXPERIMENTS DETERMINING THE PERCENTAGE COMPOSITION OF POPULATIONS GROWN ON GLUCOSE WITH RESPECT TO THE ABILITY OF INDIVIDUALS TO PRODUCE CO_2 FROM GALACTOSE. EACH EXPERIMENT REPRESENTS THE COMBINED RESULTS OF FIVE TEST PLATES

	EXPERIMENT NO. 1	TOTAL NUMBER OF COLONIES COUNTED	AVERAGE % OF POSITIVES OBSERVED
Strain Db23B	1	662	7
	2	626	9
	3	622	5
	4	625	15
	5	655	5
	6	599	7
	7	560	4
	8	685	2
	9	530	5
	10	577	13
Strain LK2G12	1	526	100
	2	480	100
	3	692	100
	4	510	100
	5	525	100

Disregarding the quantitative aspect of the problem for the moment, the important point is established that normal populations of Db23B growing on glucose contain two types of individuals which can be classified on the basis of the behavior of their colonies when grown on galactose agar test plates.

(*b*) *Data obtained with LK2G12:* Table 1 also records some representative results obtained from the examination of LK2G12 colonies grown on glucose agar. It is evident that here we are dealing with a population homogeneous with respect to the observed characteristic.

(*c*) *Validity of method:* It might be argued that whether a particular colony exhibited gas production by a crack in the agar might depend on local characteristics of the agar in which it found itself.

Two types of experiments were undertaken to examine this question. In the first type 5% glucose rather than galactose test plates were prepared and seeded in exactly the same fashion with Db23B which was

known to ferment glucose with great ease, as determined from manometric experiments on this strain in an atmosphere of nitrogen.

If the appearance of a crack immediately above a colony did depend on local imperfections, then the same general picture should be obtained in these glucose plates as was noted in the galactose test plates. In all, 11 test plates were so prepared and 2592 colonies examined. Of these only one did not show a strong positive reaction as exhibited by the typical star-shaped crack. This latter colony when isolated and examined manometrically appeared to have the non-fermentative type of glucose oxidation.

TABLE 2

EXPERIMENTS TESTING VALIDITY OF DETECTING METHOD. PERCENTAGES ARE CALCULATED TO THE NEAREST INTEGER. EACH FIGURE REPRESENTS THE AVERAGE OF FIVE TEST PLATES

EXPERIMENT NO.	% POSITIVES SUSPENSION 1	% POSITIVES SUSPENSION 2	VOLUME RATIO OF SUSP. 1:SUSP. 2 USED IN MIXTURES
1	20	48	1:1
2	4	98	1:1
3	10	67	1:1
4	10	67	1:2

EXPERIMENT NO.	COMPOSITION OF MIXTURES OBSERVED % POSITIVES	COMPOSITION OF MIXTURES CALCULATED % POSITIVES	DIFFERENCE CALCULATED − OBSERVED
1	31	34	3
2	48	51	3
3	38	38	0
4	50	48	−2

As a final test, the following experiment was performed: it was found, as was to be expected, that the per cent of gas-producing colonies obtained in a test plate originating from a positive colony on galactose was much higher than either from a negative one on galactose or a normal one growing on glucose. The percentage of positives obtained from such a colony varied from 75 to 99% depending on the length of incubation, a general rise with time being noted. Suspensions of equal density as determined by cell count were made of a normal colony from glucose and a positive one from a galactose plate. Five test plates were made from samples of each suspension to determine the percentage composition of the population. They were then mixed in 1:1 and 1:2 proportions and five galactose test plates made from samples of the mixtures. If the appearance of the cracks is truly a characteristic of the colony and not one of the locality in which it finds itself, it should be possible to calculate the percentages of positives to be found in the test plates of the mixtures from the percentage composition and proportions used of the two suspensions that went to make it up. The results of these experiments are recorded in table 2.

It is seen that in the first three experiments, within an average difference of 1.5 from the calculated value, the observed per cent positives in the test plates of the mixtures are the arithmetic means of the percentages of the two suspensions from which they originated. In the fourth experiment in which a 1:2 proportion was used, the deviation from the calculated value is 2%.

(*d*) *Measurements of enzyme activity:* As measured by gas evolution in nitrogen, both strains can equally well acquire the ability to ferment galactose if they are grown in the presence of the sugar. Evidently, then, increase in cell number is sufficient to effect the physiological change in either population. The problem is, however, whether the change will occur without this mechanism.

The results on the test plates indicate that Db23B grown on glucose consists mainly of individuals that cannot ferment galactose. On this basis it would appear probable that such a population would have to depend on cell division to change its over-all character with respect to this property by increasing through selection the proportion of individuals that can ferment the sugar. Thus a population of this nature should not "adapt" in the presence of galactose if cell division were either completely or significantly depressed.

Such experiments were performed on Db23B. To keep multiplication down to a minimum the cells were washed in $m/15$ KH_2PO_4 and resuspended in this solution with purified galactose as the only addition. Under such conditions cell counts remain constant over long periods of time. Anaerobic gas production was followed by the usual manometric methods. So long as the total cell count remained constant no change was observed from the very low rate of (endogenous) CO_2 evolution. As pointed out previously, negative results such as these have little crucial interpretive value. They do, however, become more meaningful when they are combined with the test-plate data, and the fact that this strain (in common with many others cited in the literature) invariably adapts to the substrate when allowed to proliferate in its presence.

The results obtained with LK2G12 under the same conditions are entirely different. With this strain, adaptation occurs quickly and almost explosively as may be noted from figure 2. In this experiment exactly the same experimental conditions were imposed as obtained in the experiments on Db23B. As can be seen, a very sharp change in the physiological characteristics of the population took place at the end of about 3 hrs. During this period no measurable change in the total number of individuals occurred as determined by total counts at the beginning and end of the run. These are plotted on the same graph. We have here a clear cut case of "adaptation" without the formation of new cells. It may be mentioned that this lag period of 3 hrs. is under standard conditions, reproducible,

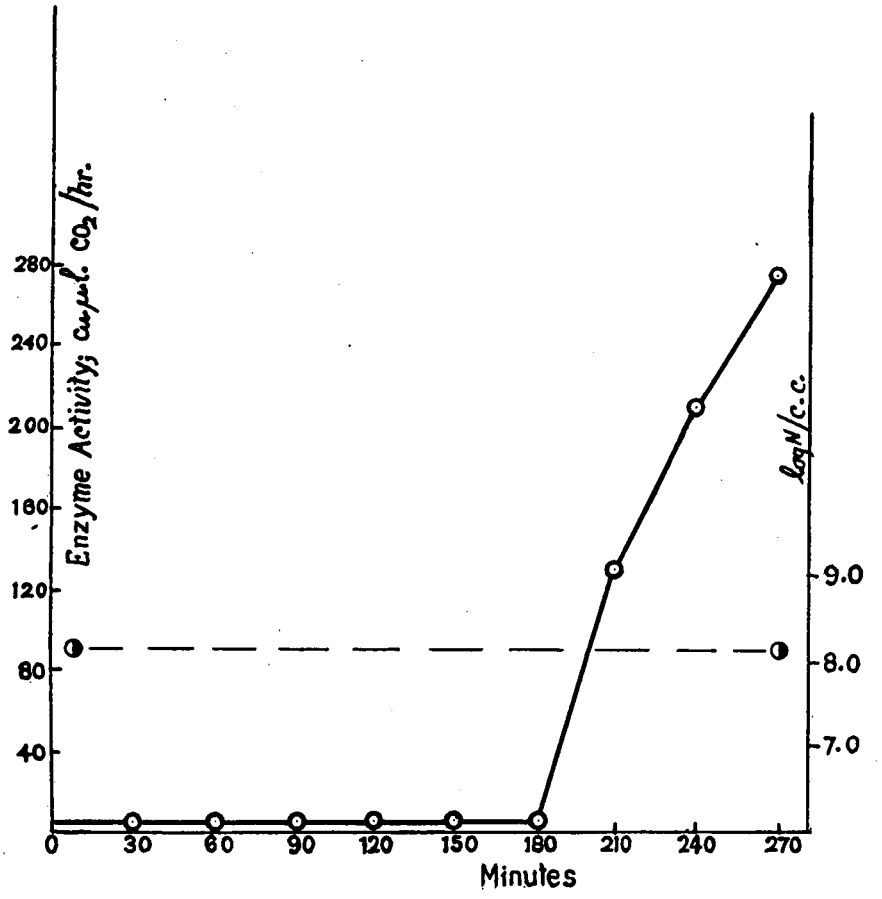

FIGURE 2

Change in physiological character of a stationary LK2G12 population in contact with galactose. ⊙ = Enzyme activity, ◑ = Log of number of cells per cubic centimeter.

and is characteristic for this strain. Other strains examined under the same conditions have longer lag periods extending up to 8 hrs.

Discussion.—These results are not only compatible with the previous test-plate examination of the phenotypic homogeneity of the populations but, in turn, the phenotypic pictures coincide well with what is known about the genetic background of the two strains employed; i.e., Db23B being principally composed of haploid cells would tend to be genetically unstable and consequently would give rise to phenotypically heterogeneous populations so long as vegetative reproduction is maintained.

These experiments offer a reasonable explanation for the apparently contradictory results obtained by various workers on the question of the rôle of

cell division in the "acclimatization" of yeast populations to galactose fermentation. Apparently Söhngen and Coolhaas,[4] as well as Euler[5] and his co-workers, were dealing with a culture of the Db23B type whereas Stephenson and Yudkin[6] probably employed a type represented by LK2G12, although with a much longer lag period.

The experiments described were specifically designed to investigate the biological mechanisms underlying populational "adaptations." As such they cannot provide solutions to the many biochemical problems of a more or less fundamental nature which have arisen during the course of the investigation. One important point may, however, be noted here which has as yet received too little attention; namely, that CO_2 evolution need not parallel galactose utilization as a source of energy for growth and maintenance. Of 34 strains of yeast examined in this laboratory, only one showed inability to grow in a medium containing galactose as sole source of carbohydrate, yet 80% of those that could use this sugar failed to ferment it. In particular the utilization of galactose by the "unadapted" culture of Db23B (glucose grown) was examined very carefully by following galactose disappearance during non-fermentative periods.

The existence of these non-fermentative oxidations of carbohydrate has been demonstrated in other forms using other sugars. Trautwein and Wiegend[13] have shown the direct oxidation of maltose by certain molds. Recently Barron and Freidemann[14] have demonstrated the oxidation of glucose and hexose-phosphates by bacteria which do not ferment glucose. As previously noted, a yeast strain possessing this type of glucose oxidation was isolated from one of our experimental glucose test plates.

With this point in mind, it is clear that the present investigation, in common with previous ones on "galactose adaptation," is not concerned with adaptation to galactose utilization *per se*, but with the development of a particular enzyme system leading to a fermentative oxidation of the sugar. The characteristics of the non-fermentative oxidations of galactose will be discussed elsewhere.

Whether it is appropriate to call the lag phenomenon observed with the LK2G12 strain in the presence of galactose an "adaptation," cannot be decided without an opportunity to examine the biochemical data in greater detail. Nor is it here possible to evaluate properly the usefulness of Karström's[10] classification of galactozymase as an "adaptive enzyme" in contradistinction to the glucose fermenting system as a "constitutive" one. Data obtained in this laboratory on the kinetics of the synthesis and destruction of the galactose fermenting system in a genetically stable population, would rather suggest that one was dealing with an enzyme system extremely unstable in the absence of its specific substrate and that what Karström calls a "constitutive" enzyme is simply one that is relatively more stable.

Whatever the case may be, the results reported here would suggest that a proper investigation into the nature of the acclimatization towards CO_2 production from galactose requires some knowledge of the genetic background of the strain being employed as well as an examination of the phenotypic homogeneity of the starting population. Unless this is done, there is the ever-present risk of unknowingly complicating the experiments by studying two distinct phenomena at the same time, one involving the competitive replacement of one phenotype by another and the other the kinetics of the enzyme substrate interaction. The latter problem can be studied safely only in a genetically stable and homogeneous population.

Summary and Conclusions.—1. A method is described involving the use of double-layered agar test plates which permits an examination of the phenotypic homogeneity of yeast populations with respect to the ability of individual members to produce gas from galactose. This method was used to elucidate the mechanism of enzymatic adaptation in a haploid and in a diploid strain of yeast.

2. Adaptation in the haploid was found to occur only in increasing populations.

3. Adaptation in the diploid could occur in the absence of cell division.

4. The necessity for a knowledge of the genetic background and phenotypic composition of initial populations is emphasized, to avoid complicating studies of enzyme synthesis with the kinetics of competitive interaction between phenotypes.

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[7] The word "new" is here used advisedly and is not meant to imply that the enzyme system formed was necessarily entirely foreign to the cell. Experimentally, due to the limitations of available techniques, it is impossible to distinguish between this situation and one where the enzyme was actually present but in too small an amount to be measured.

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